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(19)(CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Production of a Single-Gene-Encoded Immunoglobulin

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PRODUCTION OF A SINGLE-GENE-ENCODED IMMUNOGLOBULIN

ABSTRACT OF THE DISCLOSURE

Construction of a single gene encoding a signal-chain immunoglobulin-like molecule is described. This single-gene approach circumvents inefficiencies inherent in delivering two genes into a mammalian cell and in the assembly of a functional immunoglobulin molecule. It also facilitates ex vivo transfection of cells for gene-therapy protocols. The single-chain protein comprises the heavy- and light-chain variable (V_H and V_L) domains of a monoclonal antibody covalently joined through a short linker peptide, while the carboxyl end of a V domain is linked to the amino terminus of a human constant region such as γ1 Fc, through the hinge region. The single-chain protein assembles into a dimeric molecule of ~120 kDa and is secreted into the culture fluid. The single-chain immunoglobulin-like protein shows similar antigen binding affinity to that of chimeric or parental antibody and mediates ADCC. This single-gene construct approach provides a way of generating an immunoglobulin-like molecule which retains the specificity, binding properties, and cytolytic activity of a parental monoclonal antibody, and thus is a useful therapeutic and diagnostic reagent against a range of antigens, such as human carcinomas.

PRODUCTION OF A SINGLE-GENE-ENCODED IMMUNOGLOBULIN

Background of the Invention

Single-chain immunoglobulin binding fragments, or sFv, are made up of the heavy chain variable (V_H) and light chain variable (V_L) domains joined together through a short linker peptide. The sFv protein carries the antigen binding site, which confers binding specificity on the molecule. These molecules have been found to have an extremely rapid plasma and whole-body clearance rate in mice and rhesus monkeys (Milenic et al., Cancer Res. 51:6363-5371 (1991)). The sFv shows more rapid tumor penetration and more even distribution throughout the tumor mass (Yokota et al., Cancer Res. 52:3402-3408 (1992)) than a corresponding chimeric murine-human immunoglobulin. Recent studies have also shown, however, that the sFv and Fab' forms have lower equilibrium association constants (K_a) than the dimeric parental forms of the molecule; this, along with rapid clearance, has manifested itself in a lower percent injected dose per gram of sFv being deposited in a tumor site.

The sFv molecule per se, however, will not be sufficient for therapeutic use in its native (unconjugated) form. The cytolytic functions, such as ADCC and complement-dependent cytotoxicity, reside in the Fc region, which is exclusively made up of the constant-region domains of the heavy chain (C_H domains). For a native antibody to be therapeutically effective against tumors it must, therefore, carry both antigen binding site and the Fc region. It has also been shown that N-linked glycosylation of Asn-297 within the C_H2 domain is critical for binding to the Fc receptor of the human effector cells and is necessary for ADCC activity

(Tao et al., J. Immunol. 143:2595-2601 (1989), Dorai et al., Hybridoma 10:211-217 (1991) and Horan Hand et al., Cancer Immunol. Immunother. 35:165-174 (1992)). Fc-linked glycosyl residues are also implicated in complement fixation (Tao et al., J. Immunol. 143:2595-2601 (1989)). Since glycosylation of the Fc region is a characteristic of the eukaryotic system, an unconjugated antibody for therapeutic application must be produced in eukaryotic cells.

Transfection of eukaryotic cells remains highly inefficient, at best. It is all the more inefficient to develop a transfectant synthesizing a functional antibody molecule encoded by two separate genes. Currently, it is not feasible to carry out ex vivo introduction of two immunoglobulin genes simultaneously in a significant percentage of a cell population for reintroduction of the transfected cells into the host for genetic immunotherapy.

Monoclonal antibody (mAb) CC49, a murine IgG1 (1), is a second-generation monoclonal of mAb B72.3 (Colcher et al., Proc. Natl. Acad. Sci. USA 78:3199-3203 (1981)), which reacts with the tumor-associated glycoprotein TAG-72 (Johnson et al., Cancer Res. 46:850-857 (1986)) expressed on a variety of carcinomas. Murine mAb CC49 was developed by immunizing mice with TAG-72 purified by B72.3 affinity chromatography. Compared with B72.3, mAb CC49 has a higher antigen binding affinity (Muraro et al., Cancer Res. 48:4588-4596 (1988)) and targets human colon carcinoma xenografts in mice more efficiently and reduces the growth of the xenograft with greater efficacy (Colcher et al., Cancer Res. 48:4597-4603 (1988) and Molinolo et al., Cancer Res. 50:1291-1298 (1990)). Chimeric B72.3 with a human γ1 constant region has been shown to efficiently mediate antibody-dependent cellular cytotoxicity (ADCC). Results from ongoing clinical trials suggest that murine mAb CC49 is potentially a useful clinical reagent for targeting human colorectal carcinoma lesions. While the single-chain sFv form of CC49 has been shown to have important diagnostic use, what is needed in the art is a means to provide therapeutically useful single chain binding

molecules of CC49 and other clinically useful antibodies. Quite surprisingly, the present invention fulfills this and other related needs.

Summary of the Invention

The present invention provides an isolated polynucleotide molecule which encodes, under the control of a single promoter sequence a heavy chain variable region of an immunoglobulin that binds to an antigen of interest, a linker peptide, a light chain variable region from said immunoglobulin, and a human immunoglobulin Fc domain. Alternatively, the light chain variable region may be placed at the amino terminus of the polypeptide, ahead of the variable regions from the heavy chain. The resulting immunoglobulin-like molecule is capable of forming an immunoglobulin dimer and binding to said antigen. With appropriate effector functions, the single-gene-encoded immunoglobulin-like molecule is capable of mediating antibody-dependent cellular cytotoxicity against cells which express the antigen of interest. Also provided are the immunoglobulin-like polypeptides encoded by the polynucleotide sequences and host cells transfected by said sequences, as well as methods for inserting said sequences into host cells, in vivo or ex vivo.

Brief Description of the Drawings

Fig. 1 shows a schematic flow sheet for the generation of the single gene SCA$_s$C$_L$C$_H$1 of mAb CC49 and its expression construct. The template DNA (pLGCKSN49), the final single gene (SG$_s$C$_L$C$_H$1), the expression construction (pLNCS23), and a flow sheet of steps leading from the template DNA to the single-gene construct are shown schematically. CMV, human cytomegalovirus promoter; LTR, long terminal repeat; Neo,

neomycin-resistance gene; Amr, ampicillin-resistance gene; SV, simian virus 40 promoter; S, Sac II; H, HindIII.

Fig. 2 shows a schematic diagram of the dimeric protein SCA$_L$C$_H$1.

Fig. 3 shows the SDS/PAGE analysis of protein G-purified SCA$_L$C$_H$1 under nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. Lanes: M, markers (sizes in kilodaltons at left); 1 and 3, chimeric CC49; 2 and 4, protein G column purified SCA$_L$C$_H$1 secreted by transfected SP2/0 cells.

Fig. 4 shows competition assay for binding of SCA$_L$C$_H$1 (□), murine monoclonal antibody CC49 (●), chimeric CC49 (○), MOPC-21 (■), and human IgG1 (▲) were used in increasing concentrations to compete for the binding of biotinylated murine monoclonal antibody CC49 to the protein extract of the TAG-72-positive LS-174T human colon carcinoma.

Fig. 5 shows the ADCC of SCA$_L$C$_H$1, where a 24-hr ^{111}In-release assay was performed using untreated (Fig. 5A) and IL-2 (100 units/ml)-treated (Fig. 5B) human effector cells. Effector cells and ^{111}In-labeled KLE-B human endometrial carcinoma target cells were used at different effector/target cell ratios in the presence of SCA$_L$C$_H$1 (■), chimeric CC49 (▲), murine CC49 (□), and human IgG1 (△).

Description of the Specific Embodiments

The present invention provides an immunoglobulin-like molecule containing Fc functions while obviating the need for delivering two independent genes in a single cell. The invention also circumvents the problem of inefficient assembly of differentially expressed heavy and light chains into a functional immunoglobulin molecule. The present invention provides a molecule with a covalently linked V_H, V_L, and Fc domains encoded in a single gene. This immunoglobulin-like molecule takes advantage of the fact that single-chain Fv

proteins can retain the antigen-binding specificity and affinity of the original antibody, despite covalent linkage between the V_H and the V_L domains. In addition, the effector functions of the C_H domain (e.g., of human IgG1) can be maintained in a chimeric molecule.

In an exemplary embodiment of the invention described herein, a single-gene-encoded immunoglobulin-like molecule is derived from a parental chimeric (mouse-human) monoclonal antibody. A dimeric molecule which comprises the variable domains of murine antibody and the Fc region of human IgG1 is secreted by the mammalian cell transfected with the construct containing the single gene. The dimeric immunoglobulin-like molecule retains the ADCC activity and the antigen-binding specificity of the chimeric monoclonal antibody. This immunoglobulin-like molecule offers therapeutic advantages, and, by virtue of being chimeric, induces little or no human anti-murine antibody response in human patients. The single-gene construct also permits the ex vivo transfection of cells for the delivery of a tumoricidal antibody to a tumor site for gene therapy.

The preparation of single polypeptide chain binding molecules of the Fv region, single-chain Fv molecules, is described in U.S. Patent No. 4,946,778, which is incorporated herein by reference. In the present invention, single-chain Fv-like molecules are synthesized by encoding a first variable region of the heavy or light chain, followed by one or more linkers to the variable region of the corresponding light or heavy chain, respectively. The selection of appropriate linker(s) between the two variable regions is described in U.S. 4,946,778. An exemplary linker described herein is $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$. The linker is used to convert the naturally aggregated but chemically separate heavy and light chains into the amino terminal antigen binding portion of a single polypeptide chain, wherein this antigen binding portion will fold into a structure similar to the original structure made of two polypeptide chains and thus retain the ability to bind to the antigen of interest.

The nucleotide sequences encoding the variable regions of the heavy and light chains, joined by a sequence encoding a linker, are joined to a nucleotide sequence encoding antibody constant regions. The constant regions are those which permit the resulting polypeptide to form interchain disulfide bonds to form a dimer, and which contain desired effector functions, such as the ability to mediate antibody-dependent cellular cytotoxicity (ADCC). For an immunoglobulin-like molecule of the invention which is intended for use in humans, the constant regions will typically be substantially human to minimize a potential anti-human immune response and to provide approbate effector functions. In preferred embodiments, the CH1 domain is deleted and the carboxyl end of the second variable region is joined to the amino terminus of CH2 through the hinge region. The Cys residue of the hinge which makes a disulfide bond with a corresponding Cys of the light chain, to hold the heavy and light chains of the native antibody molecule, can be deleted or, preferably, is substituted with, e.g., a Pro residue or the like. Thus, the Cys residues which remain in the hinge region are those which provide disulfide linkage between two heavy chains. A schematic diagram of the resulting immunoglobulin-like dimeric molecule is shown in Fig. 2.

To prepare the polynucleotide sequence of the single-gene encoded immunoglobulin-like molecule, it is possible to utilize synthetic DNA by synthesizing the entire sequence de novo. Alternatively, it is possible to obtain cDNA sequences coding for certain preserved portions of the variable light and heavy chain regions of the desired antibody, and splice them together, by means of the necessary sequence coding for the peptide linker, which sequences are further spliced to sequences encoding the desired heavy chain constant region domains.

The resulting sequences can be amplified by utilizing well known cloning vectors and well known hosts. Furthermore, the amplified sequence, after checking for correctness, can be linked to promoter and terminator signals,

inserted into appropriate expression vectors, and transformed into hosts such as eukaryotic hosts, preferably mammalian cells which are capable of correcting processing the immunoglobulin-like chains, e.g., the SP2/0-Ag14 murine myeloma cell line. Bacteria, yeasts (or other fungi) or mammalian cells can be utilized Upon expression the single-chain binding protein is allowed to refold in physiological solution, at appropriate conditions of pH, ionic strength, temperature, and redox potential, and assemble as dimers to form the dimeric immunoglobulin-like molecules. These molecules can then be purified by standard separation procedures. These include chromatography in its various different types, e.g., affinity chromatography, known to those of skill in the art.

The thus obtained purified single-chain immunoglobulin-like binding protein can be utilized by itself, in detectably labelled form, in immobilized form, or conjugated to drugs or other appropriate therapeutic agents, in diagnostic, imaging, biosensors, purifications, and therapeutic uses and compositions. Essentially all uses envisioned for antibodies or for variable region fragments thereof can be considered for the molecules of the present invention.

Generally, it is possible to utilize the cDNA sequences obtained from the light and heavy chains of the variable region of the original antibody as a starting point. These sequences can then be joined by means of genetic linkers coding for the peptide linker. As noted above, the genetic sequence can be entirely synthesized de novo or fragments of cDNA can be linked together with the synthetic linkers.

A large source of hybridomas and their corresponding monoclonal antibodies are available for the preparation of sequences coding for the H and L chains of the variable region. Most variable regions of antibodies of a given class are in fact quite constant in their three dimensional folding pattern, except for certain specific hypervariable loops. Thus, to choose and determine the

specific binding specificity of the single-gene encoded immunoglobulin-like binding protein of the invention it becomes necessary only to define the protein sequence (and thus the underlying genetic sequence) of the hypervariable region. The hypervariable region will vary from binding molecule to molecule, but the remaining domains of the variable region will remain constant for a given class of antibody.

Source mRNA can be obtained from a wide range of hybridomas. See for example the *ATCC Catalogue of Cell Lines and Hybridomas*, 7th ed., 1992, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Hybridomas secreting monoclonal antibodies reactive with a wide variety of antigens are listed therein, are available from the collection, and usable in the invention. Of particular interest are hybridomas secreting antibodies which are reactive with tumor associated antigens, viral antigens, bacterial and fungal antigens, lymphocyte and cell adhesion antigens, and the like. These cell lines and others of similar nature can be utilized to copy mRNA coding for the variable region or hypervariable region or one may determine amino acid sequence from the monoclonal antibody itself. The specificity of the antibody to be engineered will be determined by the original selection process. The class of antibody can be determined by criteria known to those skilled in the art, and one need only replace the sequences of the hypervariable regions (or complementary determining regions). The replacement sequences will be derived from either the amino acid sequence or the nucleotide sequence of DNA copies of the mRNA.

A genetic construct comprising the isolated polynucleotide molecule of the single-gene-encoded immunoglobulin-like molecule is typically placed under the control of a single promoter. A variety of promoters and transcriptional enhances suitable for controlling and/or enhancing immunoglobulin expression are available, e.g., the human cytomegalovirus promoter, etc. DNA constructs for

expressing human immunoglobulins are described in EP patent publication EP 0 314 161, incorporated herein by reference. The expression of the immunoglobulin-like molecule can also be placed under control of other regulatory sequences which are known to those skilled in the art.

Preferred host cells are mammalian cells, grown in vitro in tissue culture, or in vivo in animals. Mammalian cells provide post translation modifications to immunoglobulin protein molecules including correct folding or glycosylation at correct sites. Mammalian cells useful as hosts include cells of fibroblast origin such as VERO or CHOK1, or cells of lymphoid origin, such as the hybridoma SP2/0-AG14 or the myeloma P3x63Sg8, and their derivatives. Transfection can be by electroporation, calcium phosphate coprecipitation, protoplast fusion, or microinjection. Following transfection the cells are incubated in nonselective medium or selective medium. After a sufficient time for cell outgrowth, the supernatants are tested for the presence of the desired immunoglobulin-like molecule by any of a variety of techniques, e.g., ELISA or the like.

The expressed and refolded single-gene-encoded immunoglobulin-like binding proteins of the invention can be labelled with detectable labels such as radioactive atoms, enzymes, biotin/avidin labels, chromophores, chemiluminescent labels, and the like for carrying out standard immunodiagnostic procedures. These procedures include competitive and immunometric (or sandwich) assays. See., e.g., U.S. Patent 4,376,110, incorporated herein by reference. These assays can be utilized for the detection of antigens in diagnostic samples. In competitive and/or sandwich assays, the binding proteins of the invention can also be immobilized on such insoluble solid phases as beads, test tubes, or other polymeric materials. For imaging procedures, the binding molecules of the invention can be labelled with opacifying agents, such as NMR contrasting agents or X-ray contrasting agents. Methods of binding labelling or imaging agents or proteins as well as binding the proteins to insoluble solid

phases are well known in the art. The dimeric immunoglobulin-like proteins can also be uses for therapy when labeled or coupled to enzymes or toxins, and for purification of products, especially those produced by the biotechnology industry, or can be used unlabeled.

Another aspect of the single-gene construct encoding the immunoglobulin-like molecule is that the construct can be efficiently and conveniently introduced into cultured human tumor-infiltrating lymphocytes (TILs). Since TILs propagate rapidly, they can be expanded and reintroduced into the host for tumor gene therapy. Delivery of the tumoricidal antibody to the tumor site will be facilitated by the preferential localization of TILs at the tumor. Introduction of cloned genes into TILs via retroviral vector has been demonstrated (Kasid et al., Proc. Natl. Acad. Sci. USA 87:473-477 (1990)), and it has recently been demonstrated that a T-cell line can secrete chimeric monoclonal antibodies (Tsang et al., J. Immunother. 13:143-152 (1993)). The single-gene approach is thus particularly attractive for *ex vivo* transfection of cells from patients for certain gene-therapy modalities, not only for cancer but also for a range of diseases in which immunotherapeutic approaches are possible.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Generation of a Single-Gene Encoding an Immunoglobulin-Like Molecule

This Example describes the generation of a single-gene-encoded immunoglobulin-like molecule, designated SCA$_4$CLCH1. The dimeric molecule is derived from chimeric monoclonal antibody CC49 in that it comprises the variable domains of the murine CC49, V_H and V_L, and the Fc region of human IgG1. It is secreted from the transfectoma and retains

the ADCC activity and the antigen-binding specificity of the chimeric monoclonal antibody CC49. The parental CC49 molecule is described in U.S. application Serial no. 07/547,336, which is incorporated herein by reference in its entirety.

The single-gene construct $SGaC_LC_H1$, encoding CC49 V_H and V_L and the entire Fc region of the human γ1 heavy chain, is shown in Fig. 1 and the immunoglobulin-like dimeric molecule is schematically presented in Fig. 2. The design of the single-chain monomeric protein included a covalent linkage of the carboxyl terminus of the V_H domain and the amino end of the V_L domain through a $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ peptide linker (Huston et al., Proc. Natl. Acad. Sci. USA 85:587-598 (1988)). Also, the carboxyl end of V_L and the amino end of the C_H2 were joined through the hinge region. In this construct, Cys^{220} of the genetic hinge was replaced by a proline residue, while Cys^{226} and Cys^{229} were retained in the functional hinge. Cys^{220} makes a disulfide bond with Cys^{214} of the *k* light chain to hold the heavy and light chains of the native antibody together. Cysteine residues 226 and 229 provide disulfide linkage between two heavy chains. The changes were brought about by primer-induced mutagenesis, as described below. Site-directed mutagenesis via amplification oligonucleotides was also used to destroy the Sac II site from the CC49 V_L exon.

The procedure used to generate the construct is presented schematically in Fig. 1. The V_H and V_L regions of CC49 and human γ1 C_H2 regions were amplified by using the construct pLGCKSN49 as the template. pLGCKSN49 was generated from the retroviral vector pLNCX by insertion of a BamHI fragment encoding chimeric heavy chain of CC49 downstream from the long terminal repeat of Moloney murine sarcoma virus and insertion of a DNA fragment encoding the chimeric light chain of CC49 at the HindIII site downstream from the human cytomegalovirus promoter.

The sequences of the oligonucleotide primers synthesized for DNA amplifications were as follows:
1 (5' V_H, coding), 5'-TCGCACAAGCTTTAACCATGGAATGGAGCTGG-3';

2 (3'V_H noncoding), 5'-CCTCCCGAGCCACCGCCTCCGCTGCCTCCGCCTCC TGAGGAGACGGTGACTGAGG-3';

3 (5'V_L coding), 5'-GCAGCGGAGGCGGTGGCTCGGGACGCGGAGGCTCGGACA TTGTGATGTCACAGTCTC-3';

4 (3' V_L, noncoding), 5'-GTCAGGAGATTTGGGCTCCGCGGCCCGTTTCAG CACCAG-3';

5 (5'C_H2, coding), 5'-GGCCGCCGAGGCCCAAATCTCCTGACAAAACTCACACA TGCCC-3';

6 (3 CH_2, noncoding), 5'-GGGGCTAAGCTTAGGCTTTCGAGATGGTTTTCTC-3'.

The overlapping complementary sequences are underlined, and the nucleotides which mismatch with the template are in lowercase letters. The sequences recognized by the restriction endonucleases are in italics. Primers 2 and 3 had a 25-bp complementing overlap and each encoded a part of the $(Gly_4\text{-}Ser)_3$ linker peptide. Similarly primers 4 and 5 carried 25-bp overlapping sequences, and together they encoded the carboxyl end of the V_L and the amino end of the hinge region. Oligonucleotides 4 and 5 were instrumental in the site-directed mutagenesis mentioned earlier. A single molecule encompassing all three amplified fragments was generated by recombinant PCR using the three amplified DNA fragments as templates and oligonucleotides 1 and 6 as 5' and 3' primers. The DNA amplification was carried out essentially as described in Horan Hand et al., Cancer Immunol. Immunother. 35:165-174 (1992), incorporated herein by reference. The final product, of ~1170 bp, was treated with HindIII. Generation of the molecule carrying the entire Fc region was facilitated by a Sac II site located ~145 bp upstream from the stop codon. By taking advantage of the enzyme site, an ~1030-bp HindIII-Sac II fragment was generated from the 1170-bp HindIII fragment. It was ligated to an ~480-bp Sac II-HindIII DNA fragment lifted from the sequence encoding the C region of the human γ1 chain present in the original template. The latter fragment encoded the C_H3 domain and the carboxyl end of the C_H2 domain. A 1520-bp HindIII fragment carrying V_H, V_L, and the entire Fc region thus generated was finally inserted in the pLNCX vector

(Tsang et al., J. Immunother. 13:143-152 (1993)) at the HindIII site located downstream from the cytomegalovirus promoter. The resulting expression construct, pLNCS23, is shown in Fig. 1.

The eukaryotic expression construct was introduced into SP2/0-Ag14 mouse myeloma cells by electroporation (Slavin-Chiorini et al., Int. J. Cancer 53:97-103 (1993)) with the Cell-Porator system (GIBCO/BRL). After electroporation, transfectants were selected in complete Dulbecco's modified Eagle's medium containing G418 (50% effective) (GIBCO/BRL) at 1.5 mg/ml.

After 2 weeks of selection, tissue culture supernatants from 20 wells were assayed by ELISA. To carry out ELISA, individual wells of the 96-well polyvinyl microtiter plates were coated with 20 µg of protein extract of either TAG-72-negative A375 human melanoma xenografts. The remainder of the assay was performed as described (Horan Hand et al., Cancer Immunol. Immunother. 35:165-174 (1992)). Fourteen well were positive for reactivity to the LS-174T human colon carcinoma cells, which express TAG-72 antigen. None of the supernatants showed reactivity to the TAG-72-negative A375 human melanoma.

Cells from the well which showed highest reactivity to TAG-72 were adapted to grow in serum and protein-free medium for further characterization. SCA$_A$C$_L$C$_H$1was purified from the protein-free culture supernatant by protein G column chromatography. Approximately 4µg of antigen-binding protein was produced per ml of the culture fluid.

For purification and physical characterization the SCAACLCH1 protein was purified from tissue culture supernatant of the producer clone grown in protein-free hybridoma medium (PFHM-II; GIBCO/BRL) by protein G affinity chromatography. Recombinant protein G-agarose was purchased from GIBCO/BRL and purification was carried out according to the supplier's recommendation. The eluted material from the column was concentrated with a Centricon 30 microconcentrator (Amicon). The concentrated material was analyzed on precast SDS/10-27%

polyacrylamide Tris glycine gels (Novex System, San Diego) with and without 2-mercaptoethanol. The proteins were visualized by staining with Coomassie blue R250.

The size and purity of protein G column-purified material were determined by SDS/PAGE analysis, as shown in Fig. 3. For comparison, chimeric CC49 was included in the analysis. Under reducing conditions, the SDS/PAGE profile of chimeric CC49 showed two distinct bands: a light-chain band of 25-27 kDa and a heavy-chain band of 50-55 kDa (lane 3). In contrast, only one distinct band was seen for $SCA_4C_LC_H1$ band showed slower migration than the heavy chain of chimeric CC49. The molecular mass of the reduced protein was ~60 kDa. Under nonreducing conditions, both chimeric CC49 and $SCA_4C_LC_H1$ appeared as individual single bands (lanes 1 and 2). The molecular masses of chimeric CC49 and $SCA_4C_LC_H1$ appeared significantly higher than expected. The presence of the intact disulfide bonds in these proteins may account for their aberrant migration. Gel filtration HPLC profiles of chimeric CC49 and $SCA_4C_LC_H1$ showed peaks consistent with molecular masses of 155 and 120 kDa, respectively. The apparent size of $SCA_4C_LC_H1$ is consistent with the estimated molecular mass of 115 kDa.

Unlike a single-chain Fv molecule, the single-chain protein of ~60 kDa assembles into a functional dimeric molecule, $SCA_4C_LC_H1$, of ~120 kDa. The disulfide bridges between the monomers are most likely made by Cys^{226} and Cys^{229}, which are retained in the hinge region (Fig. 2). Cys^{229} of the genetic hinge makes a disulfide bond with Cys^{214} of the human κ light chain to hold the heavy and the light chains of the native antibody together. Since this immunoglobulin-like molecule is deficient in its C_L region, Cys^{214} was replaced with a proline residue, lest this cysteine residue interfere with proper assembly. The substitution did not seem to affect antigen binding affinity or the effector function of the molecule.

Thus, the inefficiencies attendant with transfection and assembly of the heavy and light chains into a

functional immunoglobulin have been circumvented by constructing a single gene encoding all the domains essential for making the antigen binding site and the human IgG1 Fc region. This single gene, SCA$_L$C$_H$1, encodes a single-chain protein, SCA$_L$C$_H$1, in which V_H and V_L domains of CC49 are covalently joined together through a short linker peptide. The carboxyl end of the V_L domain of this resulting sFv fragment is in turn linked to the amino terminus of the human IgG1 Fc domain through the hinge region.

EXAMPLE II

Relative Antigen Binding Affinity of Single-Gene-Encoded Immunoglobulin-Like Molecule

This Example demonstrates via competition assays that the single-gene-encoded immunoglobulin-like CC49 SCA$_L$C$_H$1possesses almost the same binding affinity for the TAG-72 antigen as the parental murine monoclonal antibody CC49.

Competition assays were performed to compare the binding of CC49 SCA$_L$C$_H$1 murine monoclonal antibody CC49, and chimeric CC49 to the protein extracts of the TAG-72-positive LS-174T xenografts. Unlabeled immunoglobulins were used to compete with the biotinylated murine CC49 for binding to antigen. The competition assays were performed as described (Horan Hand et al., Cancer Immunol. Immunother. 35:165-174 (1992)), except that biotinylated murine CC49 was used instead of radiolabeled antibody. After the final step, the absorbance was read at 490-nm wavelength. Percent binding is the ratio of the observed absorbance to the total absorbance times 100. Total absorbance was obtained by doing the assay without the competitor. Percent inhibition was obtained by subtracting the percent binding from 100.

The results showed that all three species of mAb CC49 competed completely and the slopes of the competition

curves of the three antibodies were similar (Fig. 4). Approximately 25 nM SCA$_4$C$_L$C$_H$1, 15 nM murine monoclonal antibody, and 25 nM chimeric CC49 were required for 50% inhibition of the binding of biotinylated murine CC49 to TAG-72. Control antibodies (MOPC-21 and human IgG1) did not compete with the biotinylated antibody.

Thus, in contrast to many single-chain Fv molecules, which have lower K_a values than intact IgG, and in particular the single-chain Fv of CC49, the results of the competition assay (Fig. 4) demonstrate that SCA$_4$C$_L$C$_H$1 has almost the same affinity for TAG-72 as does the murine mAb CC49. The deficiency of the C_H1 domain in SCA$_4$C$_L$C$_H$1 did not affect its antigen binding affinity, but for some other antigen-antibody systems the absence of the C_H1 domain may alter the antigen binding affinity. The loss of the C_H1 domain may confer a certain rigidity on the binding site or may affect the spacing of binding sites crucial for antigen binding affinity.

EXAMPLE III

CC49 SCA$_4$C$_L$C$_H$1 Mediates ADCC

This Example demonstrates that the single-gene-encoded immunoglobulin-like dimeric CC49 SCA$_4$C$_L$C$_H$1 with human effector cells, mediates antibody-dependent cellular cytotoxicity against human endometrial carcinoma cells and thus is of therapeutic utility.

Chimeric monoclonal antibody B72.3 (γ1) participates with human effector cells in mediating ADCC against carcinoma cell lines that express TAG-72 on their cell surface in vitro (Primus et al., Cancer Immunol. Immunother. 31:349-357 (1990)), unlike the murine mAb B72.3 (γ1). Exposure of human effector cells to IL-2 augments this

antibody-mediated lysis of the cultured target cells (Primus et al., Cancer Immunol. Immunother. 31:349-357 (1990)).

The ADCC activity of CC49 $SCA_4C_LC_H1$ and chimeric CC49 (γ1) were compared in a 24-hr ^{111}In-release assay using KLE-B, a human endometrial carcinoma cell line which constitutively expresses the TAG-72 antigen. Human peripheral blood mononuclear cells used as effectors were cultured with or without recombinant human IL-2 (100 units/ml) for 24 hr. The remainder of the assay and calculation of percent lysis were as generally described in Hutzell et al., Cancer Res. 51:181-189 (1991), incorporated herein by reference. At each effector/target cell ratio, the ADCC activity of $SCA_4C_LC_H1$ was similar to that of chimeric CC49 (Fig. 5A). At an effector/target cell ratio of 50, cell lysis mediated by chimeric CC49 and $SCA_4C_LC_H1$ was 32% and 28%, respectively. The murine mAb CC49 and the irrelevant human IgG showed very low cytotoxicity. Exposure of the human effector cells to IL-2 (100 units/ml) substantially augmented the lytic potential of both chimeric CC49 and $SCA_4C_LC_H1$. At the highest effector/target cell ratio, target cytolysis mediated by both molecules reached 50-55% (Fig. 5B). Thus, ADCC activity of $SCA_4C_LC_H1$ is comparable to ADCC activity of chimeric CC49.

The expression, purification, and characterization of the $SCA_4C_LC_H1$ described in the foregoing Examples demonstrates that a recombinant single gene can be expressed in a mammalian cell to result in subsequent secretion of a functional immunoglobulin-like molecule. The molecule, generated by a convenient single-step transfection of the mammalian cell, shows fidelity to the antigen-binding specificity of the parental antibody and also retains its ability to mediate ADCC, a function that resides in the Fc region. This single gene approach for the generation of a functional immunoglobulin-like molecule can easily be applied to drive single-chain molecules of native therapeutic utility from other murine anti-tumor antibodies. A sFv construct flanked with appropriate restriction endonuclease sites can be

conveniently inserted in an expression cassette carrying the human Fc region. These immunoglobulin-like molecules can serve as therapeutic and diagnostic reagents against a wide range of human carcinomas and other diseases.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide molecule which codes for a single chain immunoglobulin-like polypeptide having binding affinity for an antigen, said polypeptide comprising:

(a) a first polypeptide comprising the binding portion of the light chain variable region of an antibody;

(b) a second polypeptide comprising the binding portion of the heavy chain variable region of an antibody;

(c) at least one peptide linker linking said first and second polypeptides (a) and (b); and

(d) a third polypeptide comprising the constant region domains CH2 and CH3, thereby forming a single-gene encoded single chain polypeptide having binding affinity for said antigen and capable of forming an immunoglobulin-like dimer with constant region functions.

2. The polynucleotide molecule of claim 1, wherein the constant region function is mediation of antibody-dependent cellular cytotoxicity against cells which express the antigen of interest.

3. The polynucleotide molecule of claim 1 wherein said peptide linker (c) is not from an antibody.

4. The polynucleotide molecule of claim 1 wherein said single chain polypeptide comprises one linker linking said first and second polypeptides (a) and (b) into said single chain.

5. The polynucleotide molecule of claim 3 wherein said single chain polypeptide comprises in sequence:

(i) an N-terminal polypeptide from the light chain variable region of an antibody;

(ii) a peptide linker;

(iii) a C-terminal polypeptide from the heavy chain variable region of an antibody; and

(iv) a heavy chain constant region domain.

6. The polynucleotide molecule of claim 3 wherein said single chain polypeptide comprises in sequence:

(i) an N-terminal polypeptide from the heavy chain variable region of an antibody;

(ii) a peptide linker;

(iii) a C-terminal polypeptide from the heavy chain variable region of an antibody; and

(iv) a heavy chain constant region domain.

7. The polynucleotide molecule of claim 3 wherein said heavy chain constant region domain comprises the CH2 and CH3 domains.

8. The polynucleotide molecule of claim 7 wherein said heavy chain constant region domains are substantially human.

9. A replicable cloning or expression vehicle comprising the polynucleotide molecule of any of claims 1, 2, 3, 4, 5, 6, 7 or 8.

10. The vehicle of claim 9 which is a plasmid.

11. A host cell transformed with the vehicle of claim 9.

12. The host cell of claim 11 which is a mammalian cell, a bacterial cell, a yeast cell or other fungal cell.

13. A mammalian host cell according to claim 12 which is a myeloma cell line.

14. A method of producing a single chain immunoglobulin-like polypeptide having binding affinity for an antigen and capable of forming a dimer, said polypeptide comprising (a) a first polypeptide comprising the binding portion of the light chain variable region of an antibody; (b) a second polypeptide comprising the binding portion of the heavy chain variable region of an antibody; (c) at least one peptide linker linking said first and second polypeptides (a) and (b); and (d) a third polypeptide comprising the constant region domains CH2 and CH3, thereby forming a single-gene encoded single chain polypeptide having binding affinity for said antigen and capable of forming an immunoglobulin-like dimer with constant region functions.

15. The method of claim 14 which further comprises purifying said recovered single chain immunoglobulin-like polypeptide having binding affinity for an antigen.

16. The method of claim 14 wherein said host cell is a mammalian cell, a bacterial cell, yeast, or other fungal cell.

17. The method of claim 16 wherein said host cell is a myeloma cell line.

18. The polynucleotide molecule of claim 1 wherein said first polypeptide (a) comprises substantially all of the light chain variable region, and said polypeptide (b) comprises substantially all of the heavy chain variable region.

19. The polynucleotide molecule of claim 14 wherein said first polypeptide (a) comprises substantially all of the light chain variable region, and said polypeptide (b)

comprises substantially all of the heavy chain variable region.

20. The polynucleotide molecule of claim 1, wherein the single-chain immunoglobulin-like molecule binds to a tumor associated antigen, a viral or bacterial antigen, a lymphocyte associate antigen, or a cell adhesion molecule.

21. The polynucleotide molecule of claim 21, wherein the single-chain immunoglobulin-like molecule binds to a tumor associated antigen.

22. The polynucleotide molecule of claim 21, wherein the tumor associated antigen is TAG-72.

23. The polynucleotide molecule of claim 22, which encodes $SCAAC_1C_H1$.

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Fig. 1

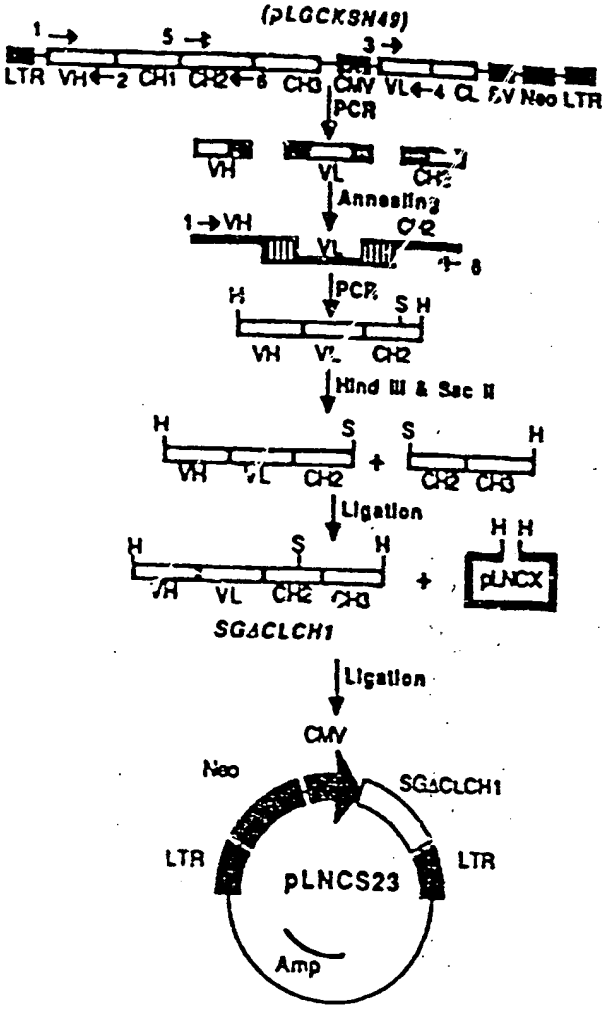

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Fig. 2

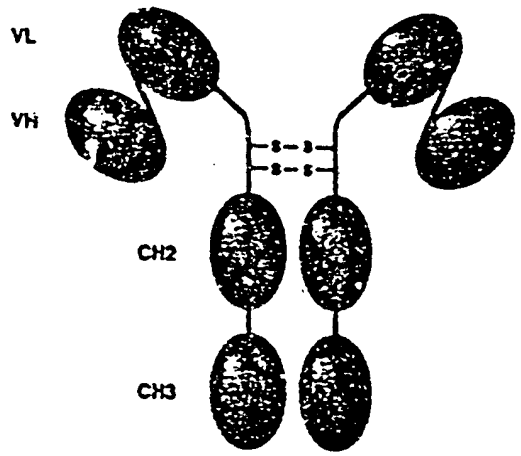

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Fig. 3

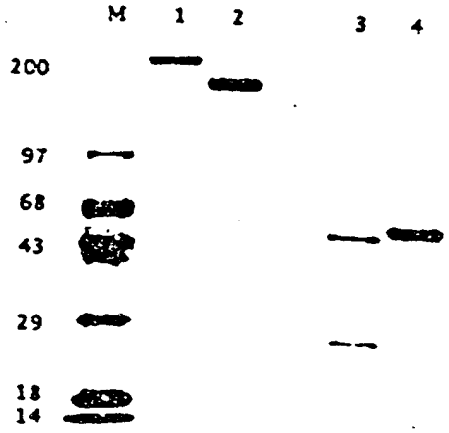

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Fig. 4

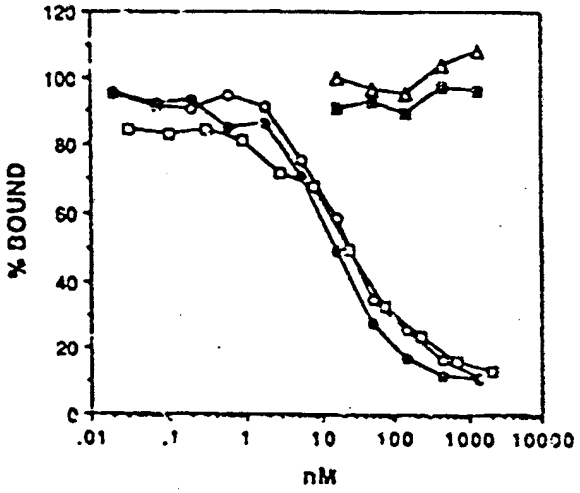
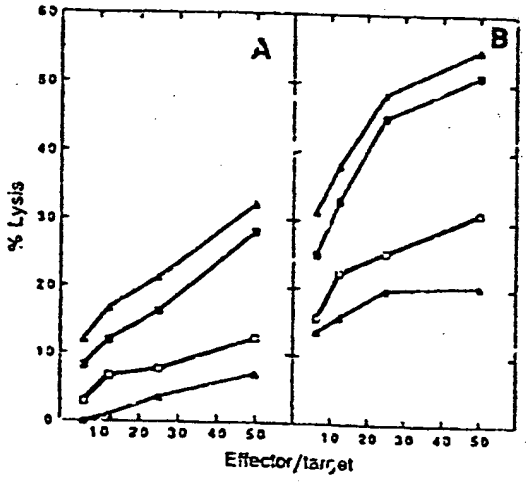


Fig. 5



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